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Proteolytic Processing of the Human Immunodeficiency Virus Envelope Glycoprotein Precursor Decreases Conformational Flexibility

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The mature envelope glycoprotein (Env) spike on the surface of human immunodeficiency virus type 1 (HIV-1) virions is derived by proteolytic cleavage of a trimeric gp160 glycoprotein precursor. Remarkably, proteolytic processing of the HIV-1 Env precursor results in changes in Env antigenicity that resemble those associated with glutaraldehyde fixation. Apparently, proteolytic processing of the HIV-1 Env precursor decreases conformational flexibility of the Env trimeric complex, differentially affecting the integrity/accessibility of epitopes for neutralizing and nonneutralizing antibodies.

The envelope glycoprotein (Env) spike on the human immunodeficiency virus type 1 (HIV-1) virion consists of a trimer of heterodimers, with three gp120 exterior Envs and three gp41 transmembrane Envs (1–6). The Env trimer mediates HIV-1 entry into host cells by binding the receptors CD4 and CCR5/CXCR4 on the target cell and fusing the viral and cell membranes (7–13).

In the infected cell, the HIV-1 Env trimer is synthesized as a precursor of approximately 850 amino acid residues and undergoes signal peptide cleavage, folding, trimerization, and glycosylation in the endoplasmic reticulum (14–16). The resulting gp160 Env precursor is transported to the Golgi apparatus, where proteolytic cleavage by host furin-like proteases produces the gp120 and gp41 subunits (17–23). The proteolytically processed, mature Env trimers are transported to the infected cell surface and incorporated into budding virions.

The Env spike is the only virus-specific target for neutralizing antibodies, which have been shown in passive protection studies to interrupt the acquisition of HIV-1-like viruses in monkeys (24, 25). The HIV-1 Envs have evolved to elicit neutralizing antibodies inefficiently, creating challenges for the development of vaccines (26). A minority of HIV-1-infected individuals, after several years of infection, generate antibodies that potently neutralize a variety of HIV-1 geographic isolates (27–31). These broadly neutralizing antibodies are directed against the CD4-binding site (CD4BS) (32–37), carbohydrate-dependent epitopes on gp120 (38–41), or peptide epitopes at the membrane-proximal external region (MPER) of gp41 (32–47).

Recognition of the mature Env trimer is a prerequisite for HIV-1 neutralization by antibodies (26, 33–35, 48–57). Although the global conformations of proteolytically cleaved and uncleaved HIV-1 Env trimers are indistinguishable at 20-Å resolution (56, 58–60), proteolytic cleavage can influence the binding of antibodies to the HIV-1 Env trimer (33, 35, 48, 61–63). For example, potent and broadly neutralizing CD4BS antibodies have been shown to recognize the proteolytically cleaved Env trimer as well as or better than the uncleaved Env trimer, whereas weakly neutralizing CD4BS antibodies efficiently recognize only the uncleaved Env trimer (33, 35, 37, 48, 62, 64). Nonneutralizing antibodies recognizing the immunodominant cluster 1 and cluster 2 epitopes on the gp41 ectodomain bound better to cleavage-deficient Env trimers than cleaved Env trimers (48). In the same study, the anti-MPER antibodies 2F5 and 4E10 recognized the uncleaved Env trimer better than the proteolytically processed Env trimer in the unliganded state. CD4 binding resulted in a large increase in the binding of the antibodies directed against the cluster 1 and cluster 2 gp41 epitopes, probably as a result of gp120 shedding. In contrast, CD4 binding only minimally increased the binding of the neutralizing 2F5 and 4E10 anti-MPER antibodies (48), although virion-binding assays suggest that these antibodies require recognition of a downstream Env conformation to achieve neutralization of HIV-1 (42, 43, 45, 49, 55, 65, 66). Thus, the conformational effects of Env proteolytic processing influence in important ways the access of antibodies to their epitopes.

We investigated the consequences of HIV-1 gp160 proteolytic cleavage on the conformation and antigenicity of the Env trimer by expressing the cleavage-competent (cl+) wild-type and cleavage-deficient (cl−) mutant Envs from two primary viruses, HIV-1_{JR-FL} and HIV-1_{AD8}. The cl− mutants have R508S and R511S changes at the site of gp160 proteolytic cleavage (17, 19). The full-length cl+ and cl− Envs were transiently expressed in HOS cells, in which we have found proteolytic cleavage of the wild-type Envs to be highly efficient compared with that in other cell types (see Fig. S1 in the supplemental material). The binding of a panel of anti-Env monoclonal antibodies (MAbs) and CD4-Ig to the cl+ and cl− Env variants on the cell surface was measured by a cell-based enzyme-linked immunosorbent assay (ELISA) (67, 68). In parallel experiments, cells expressing the wild-type AD8 and JR-FL Envs were treated with glutaraldehyde (GA) prior to measurement of ligand binding. Env-expressing cells were fixed by incubation with 5 mM glutaraldehyde for 15 min at room temperature. Glutaraldehyde activity was then halted using 25 mM gly-

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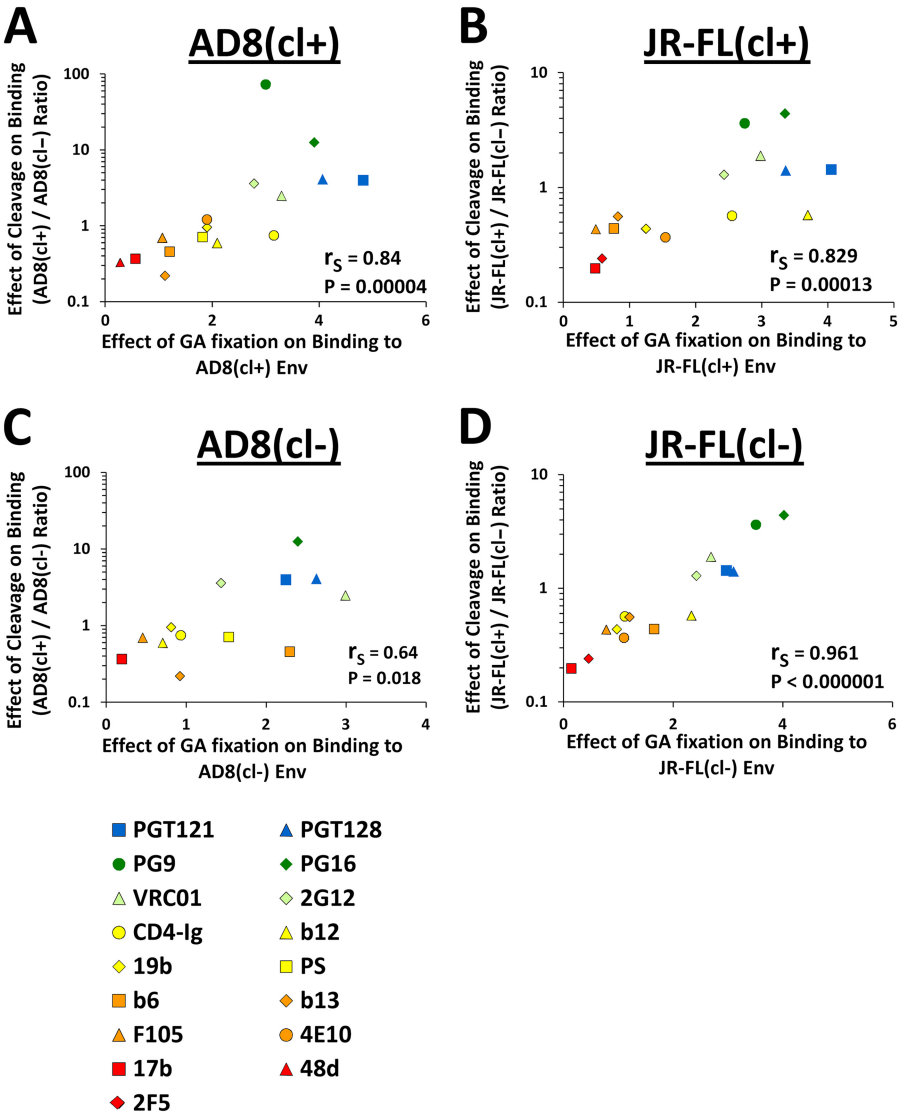

FIG 1 Relationship between the antigenic changes resulting from proteolytic cleavage and those resulting from glutaraldehyde fixation of HIV-1 Env. The effect of proteolytic cleavage on recognition of cell-surface Env trimers by the indicated ligands is represented as the ratio of ligand binding to cl+ Env to ligand binding to cl− Env. The effect of glutaraldehyde (GA) fixation on ligand recognition is represented by the ratio of ligand binding to the Env-expressing cells after GA treatment to ligand binding in the absence of GA treatment. The recognition of the JR-FL(cl+) and JR-FL(cl−) Envs by the PG9 and PG16 antibodies was, as expected (41), low but significantly greater than the background. PS, mixture of polyclonal sera from HIV-1-infected individuals. The values shown are the means derived from at least three independent experiments, each performed with two replicate samples. The Spearman rank correlation coefficient (r_S) and *P* value are shown. The averages of the coefficients of variation of the different antibodies in experiments to measure the effect of GA fixation were 11.6, 21.2, 17.4, and 14.0 for the AD8(cl+), AD8(cl−), JR-FL(cl+), and JR-FL(cl−) Envs, respectively. The averages of the coefficients of variation of the different antibodies in experiments to measure the effect of Env cleavage were 17.6 and 24.4 for the AD8(cl+) and JR-FL(cl+) Envs, respectively. Note that some antibodies are not shown for certain experiments due to low levels of binding that precluded making a reliable estimate of the ratio.

cine. Cells were subsequently washed and assayed for antibody binding using the cell-based ELISA method (see detailed protocols in the supplemental Materials and Methods in the supplemental material).

The effect of Env proteolytic cleavage on recognition by MAbs and CD4-Ig strongly correlated with the effect of glutaraldehyde fixation on recognition of the wild-type cl+ Env by these ligands (Fig. 1A and B). This relationship was observed for both the JR-FL(cl+) and AD8(cl+) Envs. Moreover, we found excellent correlations between the antigenic changes resulting from proteolytic cleavage and the antigenic effects of glutaraldehyde cross-linking of the uncleaved form of the Envs, particularly of the JR-FL(cl−) Env (Fig. 1C and D). Thus, proteolytic cleavage of the Env precursor and glutaraldehyde fixation of either the cleaved or uncleaved Env trimer exert similar effects on Env antigenicity. This implies that proteolytic processing of gp160 decreases conformational flexibility.

The AD8(cl+) and JR-FL(cl+) Envs behaved similarly with respect to the changes in the recognition of particular epitopes resulting from either proteolytic cleavage (Fig. 2A) or glutaraldehyde fixation (Fig. 2B). Likewise, GA fixation exerted similar effects on the antigenicity of AD8(cl−) and JR-FL(cl−) Envs (Fig.

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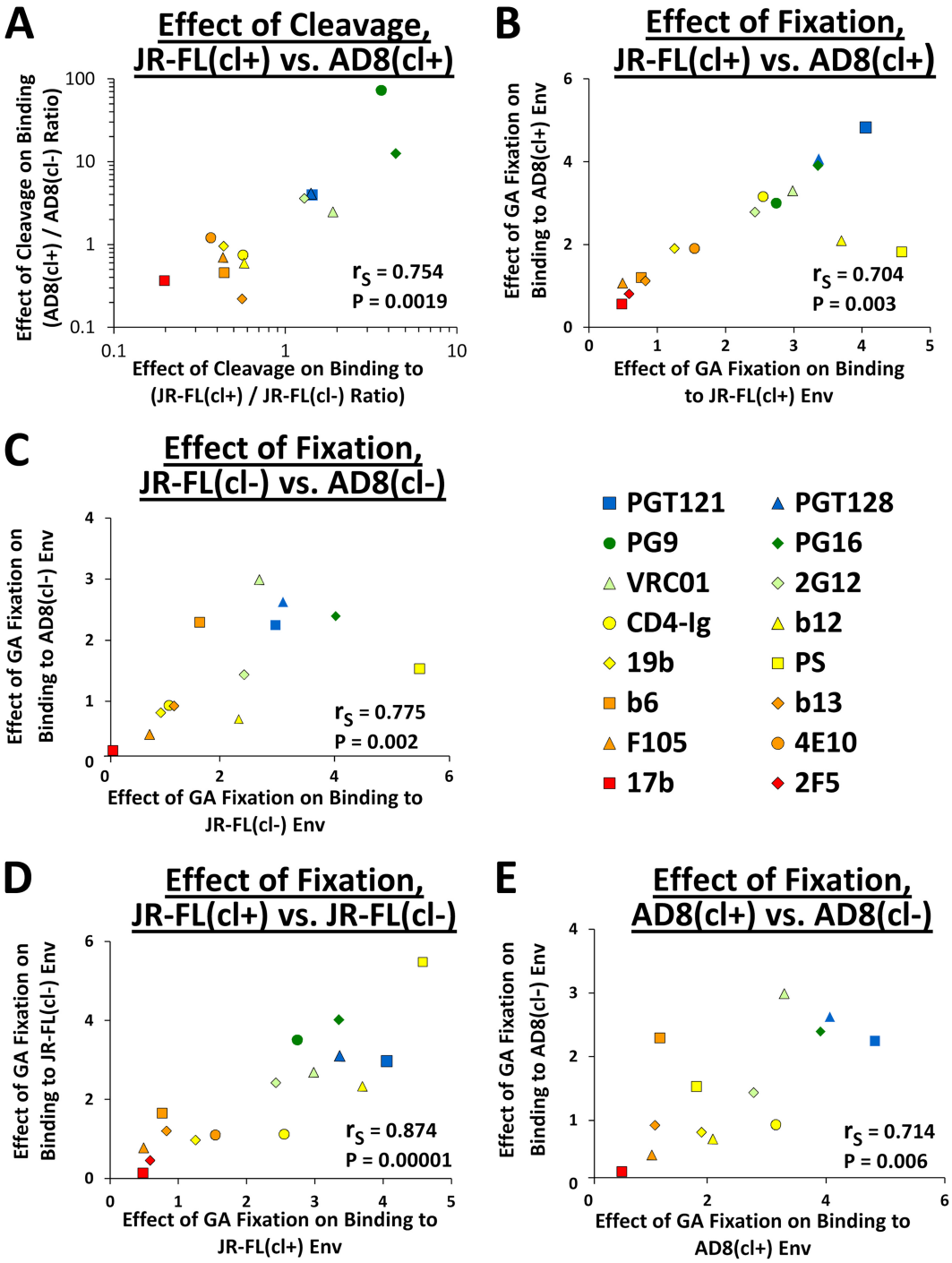

FIG 2 Comparison of the antigenic effects of proteolytic cleavage and glutaraldehyde fixation between HIV-1 variants. The effects of proteolytic cleavage (A) and glutaraldehyde (GA) fixation (B) on the binding of the indicated ligands to the AD8(cl+) and JR-FL(cl+) Envs are shown. (C) Effect of GA fixation on binding of ligands to JR-FL(cl−) and AD8(cl−) Envs. (D and E) Effects of GA treatment on the binding of the indicated ligands to the cl+ and cl− Env variants are shown. Values shown are the means derived from at least three independent experiments. The Spearman rank correlation coefficient (r_S) and the corresponding *P* value are shown.

2C). Of note, both Env cleavage and glutaraldehyde fixation decreased the binding efficiency of the 17b and 48d antibodies, which target CD4-induced (CD4i) epitopes that overlap the highly conserved coreceptor-binding site (69, 70). Such epitopes are thus minimally exposed in the cleaved Env trimer. Conversely, both Env cleavage and glutaraldehyde cross-linking increased the binding efficiency of several potently neutralizing MAbs directed against the CD4-binding site (VRC01), quaternary epitopes (PG9, PG16), and glycan-dependent gp120 outer domain epitopes (PGT121, PGT128). The observed enhancement of antibody

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binding by Env stabilization (whether by chemical fixation or as a result of proteolytic cleavage) suggests that Env conformational flexibility may reduce the integrity or accessibility of these neutralizing antibody epitopes.

The antigenicity of the cleaved and uncleaved forms of Env changed similarly upon glutaraldehyde fixation (Fig. 2D and E). These results suggest that only subtle structural differences exist between the proteolytically cleaved and uncleaved Env trimers, consistent with available low-resolution structures (56, 58–61, 63). Nonetheless, these subtle structural differences clearly exert an impact on the recognition of Env trimers by particular antibodies.

The JR-FL(cl+) and AD8(cl+) Envs exhibited similar changes in epitope recognition as a consequence of a decrease in temperature from 37°C to 4°C (see Fig. S2E in the supplemental material). The effects of proteolytic cleavage and glutaraldehyde treatment on Env antigenicity were distinct from those resulting from this temperature decrease (see Fig. S2A to D in the supplemental material). The effects of temperature and fixation on antibody binding appeared to be nonoverlapping; thus, the product of the isolated effects of a temperature decrease and glutaraldehyde fixation on antibody binding equaled the simultaneous effect of both treatments (see Fig. S3A in the supplemental material). In contrast, the effects of proteolytic cleavage and fixation demonstrated some level of redundancy (see Fig. S3B in the supplemental material). These results further support the concept that chemical fixation and proteolytic cleavage exert similar effects on the Env trimer.

In summary, our results suggest that proteolytic processing of the HIV-1 gp160 Env precursor affects multiple Env epitopes in a manner similar to that of glutaraldehyde treatment. Cross-linking by glutaraldehyde is expected to result in conformational fixation, and therefore, it is reasonable to conclude that proteolytic cleavage results in a limitation of Env conformational flexibility (Fig. 3). Of note, the antigenic changes that result from glutaraldehyde fixation are similar for the JR-FL(cl+) and JR-FL(cl−) Envs. In addition, the combined effects of GA fixation and cleavage were greater than the effects of GA fixation alone (data not shown). These observations imply that Env proteolytic cleavage allows further glutaraldehyde-mediated conformational fixation that does not occur efficiently in the context of the cleavage-deficient Env. Tighter packing of the Env subunits in the cleaved Env trimer may explain the observed relationships between proteolytic maturation and glutaraldehyde cross-linking. The resulting increase in inter- and intrasubunit stability apparently enhances the integrity/accessibility of the epitopes for potently neutralizing antibodies while helping to conceal conserved epitopes involved in coreceptor binding. That MAb binding to cleaved Env trimers is a better predictor of HIV-1-neutralizing ability than MAb binding to uncleaved Env reflects mounting evidence that more-potent HIV-1-neutralizing MAbs can bind Env spikes without inducing major conformational changes (33–35, 37, 56, 64).

The realization that proteolytic cleavage of the HIV-1 gp160 Env precursor differentially affects the binding of potently neutralizing and poorly neutralizing antibodies (33, 35, 48, 61, 63, 66) has motivated efforts to develop vaccine immunogens that faithfully mimic the native, proteolytically mature Env trimer (71, 72). The lability of the noncovalent association of gp120 with cleaved Env trimers represents a barrier to achieving this goal; one approach to overcoming this barrier has been the introduction of an artificial disulfide bond linking gp120 and gp41 (73). Our results suggest that even in uncleaved HIV-1 Envs, chemical cross-linking results in improved stabilization/exposure of neutralizing antibody epitopes and reduced integrity/accessibility of the normally cryptic epitopes for poorly neutralizing antibodies. Thus, chemical fixation might improve the ability of some trimeric Env immunogens to elicit desirable neutralizing antibodies. Moreover, glutaraldehyde cross-linking combined with analysis by a panel of anti-Env antibodies may help to identify immunogens that most closely resemble the native Env spike.

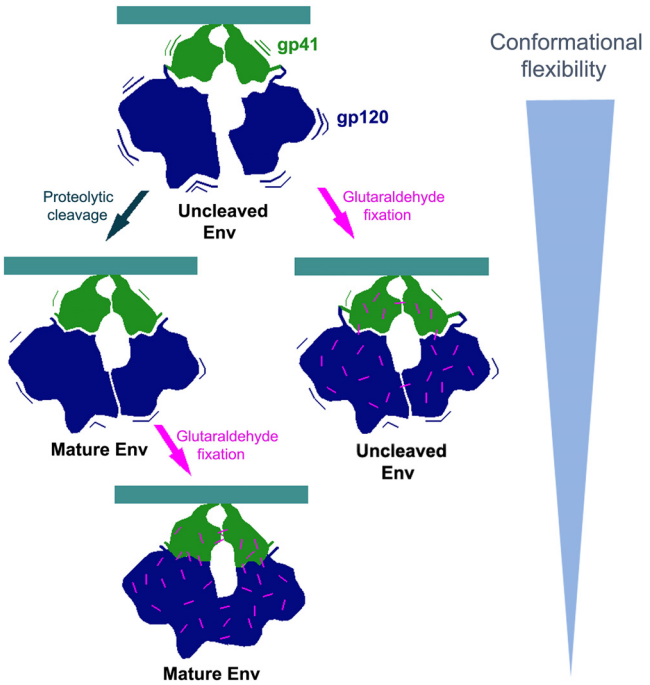

FIG 3 Model for the effects of proteolytic cleavage and glutaraldehyde fixation on HIV-1 Env trimer flexibility. The Env trimer precursor is at the top of the figure. Proteolytic processing and glutaraldehyde fixation have similar effects on Env trimer conformational flexibility, which decreases from the top to the bottom of the figure. Note that glutaraldehyde treatment of the uncleaved Env and glutaraldehyde treatment of the mature Env (magenta arrows) have similar effects on antigenicity (see Fig. 2D and E). Cross-links are represented by magenta rods.

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